Short Communication

Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element

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ABSTRACT

Iron is essential for the human being, involving in oxygen transport, energy metabolism and DNA synthesis. Iron homeostasis is tightly governed by the hepcidin–ferroportin axis, of which hepcidin is the master regulator. Excess iron is associated with various diseases including osteopenia and osteoporosis, which are closely related to the alternation of the endogenous estrogen level. To verify the biological effect of estrogen on iron metabolism, we established a mouse model of estrogen deficiency by ovariectomy. We demonstrated that the hemoglobin content and serum iron level decreased, whereas the tissue iron level in liver and spleen increased in the ovariectomized mice. Moreover, the transcription of hepatic hepcidin was elevated in ovariectomized mice compared to the control mice. We further demonstrated that there was an estrogen response element (ERE) in the promoter region of the hepcidin gene. The assay using the luciferase reporter system confirmed the existence of a functional ERE in the hepcidin promoter, as the estradiol treatment reduced hepcidin expression in cells transfected with ERE-intact construct, with no response to estradiol in cells transfected with ERE-devoid construct. In conclusion, estrogen greatly contributes to iron homeostasis by regulating hepatic hepcidin expression directly through a functional ERE in the promoter region of hepcidin gene. These findings might help build a better understanding towards the etiology of postmenopausal osteoporosis accompanied by excess tissue iron (such as iron retention of osteoclasts in bone) under estrogen deficiency.

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1. Introduction

As an essential element, most iron is stored in the hemoglobin of red blood cells, and the rest is maintained in liver, spleen and bone marrow. General iron metabolism is synergistically controlled by the hepcidin–ferroportin axis, hepcidin undertaking a leading role in this regulation. Hepcidin reduces iron absorption from the intestine, while inhibiting iron egress from macrophages (macrophages in bone are specified as osteoclasts) (Ganz, 2004, 2005). Increased hepcidin expression causes iron deficiency anemia; in contrast, decreased hepcidin expression leads to body iron overload (Ganz and Nemeth, 2011). Iron overload is recognized as a risk factor for cancers, neurodegenerative diseases and arthropathy (Ganz and Nemeth, 2011; Lehmann et al., 2006; Weinberg, 2006). Meanwhile, iron

overload is also considered as a risk for osteopenia and osteoporosis (Haidar et al., 2011; Valenti et al., 2009; Weinberg, 2008). Abnormal iron status is associated with disorders in bone metabolism. In patients with hereditary hemochromatosis, 25% patients were diagnosed with osteoporosis and 41% of them developed osteopenia (Valenti et al., 2009). In accordance with clinical observations, HFE deficient male mice, a mouse model of hemochromatosis with severe iron overload, developed osteoporosis with low bone mass and alterations of bone microarchitecture (Guggenbuhl et al., 2011).

Osteoporosis is a common bone disease among the postmenopausal women with estrogen deficiency as a result of menopause. Precious epidemiological studies have demonstrated that the postmenopausal women harbor a higher level of body iron than premenopausal women (Jian et al., 2009). Similar to clinical observations, Liu and his colleagues documented iron accumulation in the ovariectomized (OVX) rats, a model of postmenopausal osteoporosis (Liu et al., 2006). In contrast, a pilot study suggested that iron chelator could prevent bone loss induced by excess iron in OVX rats (G. Liu et al., 2008). Although the current evidence reveals a close interaction between estrogen signaling and iron homeostasis, the molecular mechanism implicated in this interaction is still elusive. To fill this knowledge gap, in the current study we further elucidated the mechanism responsible

Abbreviations: BMP6, bone morphologic protein 6; C/EBP, CCAAT/enhancer binding protein; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response element; GPR30, G-coupled protein 30; OVX, ovariectomized; Pro, the reporter vector with deletion of the putative ERE; Pro + ERE, the reporter vector with the putative ERE; RBC, red blood cell; Sham, sham-operated.

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for estrogen-mediated actions on iron metabolism by establishing a mouse model devoid of estrogen through ovariectomy. We observed reduced hemoglobin and serum iron and correspondingly increased tissue iron in liver and spleen due to estrogen deficiency in mice. Hepcidin was increased in the liver of ovariectomized mice compared to the control. Consistent with the observations *in vivo*, a functional estrogen response element (ERE) was identified in the promoter region of hepcidin gene, and 17β -estradiol was demonstrated to reduce hepcidin level in hepatocytes via direct transcriptional inhibition of its gene expression.

2. Material and methods

2.1. Animal experiments

All animal care and surgical procedures were approved by the Animal Ethics Committee at RCEES, Chinese Academy of Sciences (CAS). 16-week-old female C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China) and housed in the central animal facility of the Institute of Genetics and Developmental Biology, CAS. After 1 week of adaption, the mice were randomly divided into 2 groups with 6 in each group ($n = 6$). One group of mice were bilaterally ovariectomized (OVX), and the other group of mice were sham-operated (Sham) following the surgical procedure described in a previous study (Jilka et al., 1992). After 6 weeks, these mice were sacrificed. Blood was collected with a portion for CBC analysis and the rest for serum extraction. The specimens of liver, spleen and uterus from each mouse were individually collected and weighed. A small fraction of each liver was quickly frozen in liquid nitrogen and stored at -80°C for future analysis.

2.2. Iron parameters

Serum iron was determined by the serum iron detection kit (Nanjing Jiancheng Bioengineering Institute). The hepatic and splenic iron content was assessed as previously described (S. Liu et al., 2007, 2008).

2.3. Total RNA isolation and PCR analysis

Total RNA was extracted from liver with Trizol (Invitrogen) following the protocol provided by the manufacturer. Primer sequences for PCR reaction were as follows: hepcidin: 5'-CTGAGCAGCACCTAT CTC-3' (forward) and 5'-TGGCTCTAGGCTATGTTTGC-3' (reverse); HPRT: 5'-GCTTGCTGGTGAAAGGACCTCTCGAAG-3' (forward) and 5'-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3' (reverse). HPRT was used as the internal control. Bands of agarose gel electrophoresis were analyzed by Image J software.

2.4. Cell culture

Hepatocarcinoma cell line SMMC-7721 cells (purchased from the Shanghai Cell Bank of Type Culture Collection of CAS) were cultured in 1640 medium (Hyclone), supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Gibco). Cell experiments were similar to the standard instruction described in our recent publications (Liu et al., 2011; Qu et al., 2012).

2.5. Vector construction of hepcidin promoter with putative ERE

Using the web-based software Dragon ERE Finder version 3.0 (Bajic et al., 2003), we identified a potential ERE (5'-AGTCA-GGC-TGTCC-3', -1244/-1232) in the promoter region of human hepcidin gene. Two DNA fragments, one with the putative ERE (-1574/+73) and the other with deletion of the putative ERE (-1231/+73), were cloned from hepcidin promoter followed by insertion into the pGL3-promoter

luciferase reporter vector (Promega), respectively. The construct with the putative ERE was referred to Pro + ERE, and the construct with deletion of the putative ERE was named Pro. The two constructs were verified by sequencing.

2.6. Luciferase assays

SMMC-7721 cells were seeded at a density of 1×10^5 cells/ml in 24-well plates. Cells were then co-transfected with 0.8 μg of target constructs (Pro + ERE or Pro) and 80 ng of Renilla luciferase plasmid by Lipofectamine 2000 (Invitrogen) according to the instruction provided by the manufacturer. After 6 h, culture medium was replaced with phenol red-free 1640 containing 5% charcoal-treated fetal bovine serum (endogenous estrogen-free) and then treated with 1×10^{-7} M 17β -estradiol (E2) or an equal amount of ethanol for 24 h. Cells were subsequently collected in lysis buffer after washing by PBS twice, and the cellular extracts were analyzed for luciferase activity using the Dual-luciferase reporter assay system (Promega). The relative luciferase activities were normalized to those of Renilla luciferase.

2.7. Statistical analysis

The data were presented as mean $\pm$ standard error (SE). Statistical analysis was performed by independent *t*-test or one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Increased body weight and tissue weight under estrogen deficiency

To evaluate the establishment of the estrogen-deficient mouse model, we first examined the pathophysiological alternations of the mice with ovariectomy. The uterus weight in the OVX group was markedly decreased by 63.4% compared to that of the Sham group ($p = 0.001$, Fig. 1A). Consistent with previous studies (Mattace Raso et al., 2009; Thompson et al., 1995), the OVX mice developed greater body weight than the Sham control mice ($p < 0.05$, Fig. 1B). The gain in body weight after ovariectomy is presumably largely attributed to increased body fat because of the hyperphagia-induced obesity (Iwasa et al., 2011; Thompson et al., 1995). The liver weight was increased by 15.3% in the OVX mice compared to the control ($p < 0.05$, Fig. 1C), which might be caused by excessive hepatic fat accumulation in OVX animals (Cote et al., 2012). After adjusting with its corresponding body weight for each group, the liver index (liver weight/body weight) was similar between the OVX mice and the Sham control group (0.056 VS 0.057). The spleen weight was also pronouncedly increased in the OVX mice compared to the control ($p < 0.05$, Fig. 1D), with a greater spleen index (spleen weight/body weight) for the OVX group than the Sham group (0.0035 VS 0.0042, $p = 0.063$). These data suggested that ovariectomy-caused estrogen deficiency caused a series of pathophysiological changes in the animals, proving the establishment of the mouse model with estrogen deficiency.

3.2. Altered tissue iron distribution and reduced hemoglobin content upon estrogen deficiency

We assessed systemic iron homeostasis in mice under estrogen deficiency induced by ovariectomy. As shown in Fig. 2A, the level of serum iron was reduced by 14.0% in the OVX group compared to the Sham group. This observation was similar to the study performed on rats, as the OVX rats had reduced serum iron and estradiol treatment could reverse this situation (Mattace Raso et al., 2009). The hepatic and splenic iron levels were increased by 26.0% and 9.5%, respectively, in the OVX mice compared to the control (Fig. 2A). Considering the total iron in liver and spleen, iron levels were remarkably increased by 54.6% and 45.1%, respectively, in the OVX group in

Fig. 1. The changes of body and organ weight in the mouse model with ovariectomy. Uterus weight (A), body weight (B), liver weight (C), and spleen weight (D) in the OVX group and the Sham group after 6 weeks of surgery. The data were presented as mean $\pm$ SE (n=6).

comparison with the control group (Fig. 2B). These observations indicated that the defect in endogenous estrogen induced the deregulated iron metabolism, leading to iron retention in tissues with reduced serum iron.

Since there were significant changes for overall iron homeostasis under estrogen deficiency, we examined the hemoglobin biosynthesis through the CBC analysis. As presented in Fig. 2C, there was a decrease in hemoglobin content in the OVX group compared to the control group, as a result of the decreased serum iron described above (Fig. 2A). The red blood cell (RBC) count and hematocrit were consequently reduced in the OVX animals (Fig. 2C). Serum iron is the source for erythropoiesis, and the hemoglobin content consequently shrinks upon reduced iron supply in serum (Ganz and Nemeth, 2012). Thus, these data suggested that estrogen was largely involved in governing iron homeostasis, and alteration of endogenous estrogen level could break the balance of iron metabolism in the body, such as iron supply from serum to erythropoiesis in bone marrow.

3.3. Elevated hepcidin expression in liver devoid of endogenous estrogen

The changes of body iron content implied that the master regulator of iron homeostasis, hepcidin, could be regulated upon estrogen deficit. Hepcidin inhibits iron uptake into the plasma from three main sources of iron, dietary absorption in the duodenum, egress of recycled iron from macrophages (including osteoclasts) and release of stored iron from hepatocytes (Ganz and Nemeth, 2012). We thus examined the hepatic hepcidin expression in mice, and observed a remarkable increase of hepcidin expression in the OVX group compared to the control (Fig. 3A), evidenced by the end-point PCR analysis. The quantified data indicated a near 2-fold increase of the hepcidin level in the OVX mice compared to the control (Fig. 3A, $p < 0.05$).

The elevated hepcidin expression under estrogen deficiency in mice therefore accounted for the decrease of serum iron along with the increase of hepatic and splenic iron in the OVX mice as discussed above. The results from the current study are consistent with an epidemiological observation, demonstrating that serum hepcidin is

higher in postmenopausal women than premenopausal women (Galesloot et al., 2011). The mechanism underlying the increased hepcidin expression upon estrogen deficiency has not been recognized thus far. A previous study suggested that this may be due to increased level of inflammatory cytokines upon estrogen deficit (Jilka et al., 1992). However, in addition to inflammation-stimulated upregulation of hepcidin, other possible mechanisms may deserve further investigation.

3.4. Hepcidin transcription was regulated by estradiol through a functional ERE within hepcidin promoter

As described above, estrogen deficiency exerted a significant impact on hepatic hepcidin expression. We speculated that estrogen might have a direct role in regulating the transcription of hepcidin expression in hepatocytes. There are at least four pathways coupled to estrogen signaling. The canonical pathway is ligand-dependent, in that estrogen-activated estrogen receptors (ERs) bind to ERE in the promoter and then activate or suppress gene expression for certain target genes. In the absence of estrogen, ERs could be activated by growth factors and bind directly to ERE, which is ligand-independent pathway. There are also ERE-independent pathway and cell membrane signaling pathway (Hall et al., 2001). The consensus sequence of ERE is composed of palindromic half-sites intervened by three nucleotides (5'-GGTCA-nnn-TGACC-3'). However, other EREs may have one or more base deviations from this sequence (Klinge, 2001). We identified a potential ERE (5'-AGTCA-GGC-TGTCC-3', -1244/-1232) in the promoter region of human hepcidin gene using the software Dragon ERE Finder version 3.0 (Bajic et al., 2003) (Fig. 3B). To test its existence as a functional ERE, we constructed two reporter vectors with putative ERE (Pro + ERE) and one without it (Pro) (Fig. 3B). The transcriptional activity of Pro + ERE upon E2 was reduced by approximately 20% compared to those without E2 treatment (Fig. 3C, $p < 0.01$). However, the response to E2 was completely lost for Pro, i.e. E2 treatment did not affect the transcriptional activity in the construct without the putative ERE (Fig. 3C). These results demonstrated that the predicted ERE (-1244/-1232) functioned in response to E2 treatment, and this ERE transcriptionally suppressed hepcidin expression through the canonical pathway of estrogen signaling, consistent with the observations as discussed in Fig. 3. Moreover, it is worth noting that the transcription activity of Pro was largely reduced compared to that of Pro + ERE (Fig. 3C, $p < 0.001$) independent of E2 treatment, suggesting that the region upstream of the ERE contributed to hepcidin transcription. CCAAT/enhancer binding protein (C/EBP) was demonstrated to bind this upstream region (Fig. 3B), and C/EBP could activate hepcidin expression in hepatocytes (Courselaud et al., 2002).

This finding is supported by a recent study, which demonstrated that the transcription of hepcidin was suppressed by E2 treatment in hepatocytes, HuH7 and HepG2 cells, and this down-regulation could be blocked by the treatment of the E2 antagonist ICI 182780 (Yang et al., 2012). In the current study, the newly recognized ERE was located on -1244 to -1232 from transcription start site in the promoter region of hepcidin gene (Fig. 3B). This location differs from the one between -2474 and -2462 reported by the previous study (Yang et al., 2012). However, Ikeda and colleagues recently described different findings that the OVX mice revealed decreased hepcidin expression in livers, which was attributed to another mechanism of estrogen signaling in regulating hepcidin expression through G-coupled protein 30 (GPR30)-bone morphologic protein 6 (BMP6)-dependent pathway (Ikeda et al., 2012). The classical ERs and GPR30 are co-expressed in many types of cells and tissues, and the combined effect of estrogen on ERs and GPR30 is distinct, depending on a manner of synergism or antagonism (Prossnitz and Maggiolini, 2009). These observations reported by Ikeda et al. (2012) differ from our results in hepcidin expression and serum iron alternation in OVX mice. We speculate that the primary reason for those conflicting results is the time of

Fig. 2. The iron status and the RBC parameters in mice with ovariectomy. (A) Serum iron, hepatic and splenic iron content in the OVX group and the Sham group. (B) Total liver iron and total spleen iron content in both groups. (C) Hemoglobin content, red blood cell count and hematocrit in mice. The data were presented as mean ± SE (n = 6).

ovariectomy treatment in mice. We checked hepatic hepcidin expression and iron status of the mice at 6 weeks after ovariectomy. The major reason for choosing this time point is because biological effects induced by estrogen deficiency need at least 4–6 weeks to be reflected in animals. For example, most studies in evaluating the ovariectomized models for postmenopausal osteoporosis are performed over 1 month after ovariectomy (G. Liu et al., 2006, 2008; Sottile et al., 2004; Thompson et al., 1995). Considering iron metabolism upon certain chronic physiological alternation, the end-point of iron-related studies are normally set over 1 month after ovariectomy when using ovariectomized models (Ikeda et al., 2012; Liu et al., 2006; Mattace Raso et al., 2009). Moreover, a previous study by Ramos et al. has demonstrated that hepcidin expression were distinct in response to long-term (3 weeks) or acute (1 day) iron loading (Ramos et al., 2011). To this end, we decided to address a long-time effect of chronic estrogen deficiency on systemic iron homeostasis. Therefore, to closely reflect the changes of body iron parameters, we carried out the experiment in mice 6 weeks after ovariectomy. Hepcidin expression in response to estrogen-deficiency in vivo dramatically alters along with time. In the early stage, hepcidin expression increases in response to the dramatic reduction of estrogen, coupled to increased tissue iron and decreased serum iron level as shown in Fig. 2. Thereafter, in order to compensate for the status of iron-imbalance, particularly when animals develop anemia due to the long-term iron deficiency in serum and limited iron

supply for erythropoiesis, hepcidin expression is driven to decrease to obtain more iron by the demand of erythropoietic activity after a longer time of ovariectomy (Ganz, 2003; Ganz and Nemeth, 2012; Ikeda et al., 2012; Pak et al., 2006). This could be the mechanism interpreting the distinct observations in studies, and it thus needs further continuous longitudinal studies to confirm these findings.

Hepcidin is a 25-amino acid peptide mostly synthesized by hepatocytes (Park et al., 2001). Its transcription is activated by iron, inflammatory cytokines and BMPs, and repressed by anemia and hypoxia (Andriopoulos et al., 2009; Ganz, 2004; Ganz and Nemeth, 2012; Nemeth et al., 2004a). Hepcidin is the only known master hormone modulating systemic iron homeostasis, and ferroportin is its receptor, an iron exporter found in duodenal enterocytes, macrophages, osteoclasts and hepatocytes (Donovan et al., 2005). Upon binding to hepcidin, ferroportin is internalized and degraded via ubiquitin-dependent proteasomal degradation, leading to decreased iron egress from the above mentioned cells (Nemeth et al., 2004b). Hepcidin defect leads to iron retention in macrophages, hepatocytes and osteoclasts. Our own recent results suggested that iron overload could impair bone strength through tumor necrosis factor- α secretion which facilitates osteoclast differentiation and promotes bone-resorbing activity (unpublished data, submitted). In the current study, we demonstrated that estrogen conferred transcriptional repression to hepcidin expression through the ERE signaling (Fig. 4). Estrogen reduced hepatic hepcidin level,

Fig. 3. Hepcidin expression in mice and identification of ERE in hepcidin promoter. (A) Hepcidin mRNA expression in livers in both groups reflected by RT-PCR analysis. The bar graph shows the quantified data for hepcidin expression relative to HPRT in mice. The data were presented as mean $\pm$ SE (n = 5). (B) The localization of putative ERE in hepcidin promoter region. The potential ERE was predicted (labeled and marked with underline) using the Dragon ERE Finder version 3.0. Forward arrows indicate the start sites of the two fragments (–1574/+73 with the putative ERE and –1231/+73 without the putative ERE, respectively), which were cloned to assess the efficiency of ERE. (C) Relative luciferase activities. The pGL3-based constructs containing with ERE (Pro+ERE,) or without ERE (Pro) were co-transfected with Renilla luciferase plasmid into SMMC-7721 cells. Relative luciferase activities were normalized to those of the Renilla luciferase. The data were presented as mean $\pm$ SE (n = 4–5).

Fig. 4. A schematic delineating the mechanism responsible for estrogen-mediated regulation on iron metabolism. Estrogen inhibits hepatic hepcidin (Hepc) transcription directly through the ERE signaling. Increased hepcidin would reduce iron uptake from intestine and iron release from macrophages in various organs via the hepcidin-ferroportin (FPN) axis. This leads to elevated tissue distribution, such as increased iron concentration in osteoclasts, causing enhanced bone-resorbing activities. Whether estrogen exerts direct effect on erythropoiesis is currently elusive.

leading to elevated iron absorption and egress through the hepcidin–ferroportin axis; whereas hepcidin concentration would be elevated as a result of estrogen deficiency, resulting in decreased serum iron and increased tissue iron burden (Fig. 4).

Iron is closely linked to bone metabolism, while excessive iron is recognized as a risk factor for osteoporosis (Guggenbuhl et al., 2005; Haidar et al., 2011; Valenti et al., 2009; Weinberg, 2008). There is an elevated iron level in postmenopausal women compared to premenopausal women (Jian et al., 2009), suggesting that extra iron plays a crucial role in provoking postmenopausal osteoporosis. A variety of studies have demonstrated that extra iron burden would repress osteoblastogenesis, proliferation and mineralization (Messer et al., 2009; Yamasaki and Hagiwara, 2009; Yang et al., 2011), and enhance osteoclast differentiation and provoke osteoclastic function (Ishii et al., 2009). Thus, it could be concluded that iron surplus presumably hampers bone formation and induces bone loss. The association between iron status change and estrogen defect coupled to hepcidin upregulation suggests promising approaches to treat estrogen-related disorders, such as postmenopausal osteopenia and osteoporosis. For instance, tissue-targeted iron chelator may be helpful to treat postmenopausal osteoporosis. To this end, a recent study demonstrated that the bone-targeted chelator could protect against bone loss related to iron accumulation in OVX rats (Liu et al., 2008a).

To summarize, we demonstrated that estrogen is implicated in modulating iron homeostasis by governing hepatic hepcidin expression. Estrogen confers transcriptional inhibitory effect on hepcidin expression directly through a functional ERE in the promoter region of hepcidin gene. Upon estrogen deficiency induced by ovariectomy in mice, there was a great increase in hepatic hepcidin level, leading to serum iron level decreased and tissue iron increased. Thus, estrogen-associated disorders, such as postmenopausal osteoporosis, could practically be attributed to hepcidin defect associated with tissue iron retention (such as in osteoclasts in bone). This study also implies that targeting the estrogen–hepcidin signaling might prove to be a promising strategy for restraining iron-stimulated bone resorption in postmenopausal osteoporosis.

Authors' contributions

SL, HR and HJ conceived and designed the study. YH, SZ, JL, GQ, JH and LW carried out experiments. YH, SZ and SL analyzed the data. JL, GQ, JH and LW contributed reagents and materials. YH and SL wrote the paper. All authors read and approved the final manuscript.

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